Eastern equine encephalitis in a horse from southwestern Ontario

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A number of viruses can cause fatal neurological disease in horses, including rabies virus, equine herpesvirus-1, and certain arboviruses. Several arboviruses cause neurological disease in horses in Canada either naturally (e.g., eastern equine encephalitis (EEE), western equine encephalitis (WEE), or snowshoe hare virus) or experimentally (e.g., Powassan virus) (1,2). Neurological disease due to EEE virus can be severe, with a mortality of 75% to 90% (1). This virus is a member of the genus Alphavirus of the family Togaviridae, distinct from other North American alphaviruses, such as WEE virus and the exotic Venezuelan equine encephalitis (VEE) virus, but more closely related to Highlands J (HJ) virus, a member of the WEE virus complex (3).

Antibodies to EEE virus have been documented in passerine birds migrating to Long Point, Ontario (4), and Prince Edward Point, Ontario (5); and EEE virus was isolated from a bird at Long Point in 1961 (4). One outbreak of EEE has been reported in horses in Ontario. In 1938, 12 horses near St. George, Ontario, were afflicted with encephalomyelitis, and EEE virus was isolated from the blood of one of them (6). In the same year, 6 or 7 suspected cases of EEE were observed in the area of St. Catharines, Ontario. An outbreak of EEE occurred in 1972 in the Eastern Townships of Quebec (7,8). Enzootic foci of EEE infection occur in upstate New York, southwestern Michigan, and northeastern Indiana, as well as in the Atlantic seaboard and Gulf Coast states (8).

The EEE virus usually cycles between migratory passerine birds and the ornithophilic mosquito *Culiseta melanura* (3,8). Infected birds also infect *Coquillettidia perturbans*, a mosquito that avidly feeds on mammals, including humans (3,8). The virus may overwinter in mosquito larvae (3); however, it is likely reintroduced each spring by infected migrating birds (3,4). Mosquito vectors usually breed at the edge of large swamps with permanent fresh water (3). When rainfall is high in the preceding fall and in June and July, swamp circumference and mosquito numbers increase (9). Animals, other than birds, are then bitten. The same swamp may not have infected mosquitoes in subsequent years (3).

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Deaths of ring-necked pheasants in localized areas have been associated with endemic EEE virus (2,3). Humans, horses, suckling pigs, and calves are also susceptible to infection and may develop clinical encephalitis (3), but they are considered "dead-end" hosts in the transmission cycle (2). Unlike birds, mammals rarely develop an EEE viremia sufficient to infect mosquitoes (3).

Infection in the horse may produce either clinical or subclinical disease. The most common signs are depression, fever above 39.4°C, circling, chewing, progressive ataxia, paralysis, anorexia, and stupor. Head pressing, blindness, and convulsions can occur (1–3). A diagnosis of EEE is confirmed by virus isolation from the brain. A presumptive diagnosis of EEE virus infection can be made with a fourfold change in antibody titer between paired sera. Where only a single serum sample is available, a hemagglutinating-inhibiting antibody titer >1:40 or a virus neutralizing antibody titer >1:10 implies previous infection in unvaccinated horses (8).

We describe here the first isolation of EEE virus from a horse in Ontario since 1938. The mare, which died with signs of meningoencephalitis, lived near Woodstock in southwestern Ontario and had not travelled outside the region.

In mid-August 1992, a 5-year-old, Belgian mare was examined because of neurological signs of acute onset. She appeared blind; pressed her head into fence posts, gates, and the manger of the stall; lost coordination; and had a flaccid lower lip. Her rectal temperature was 40°C. She became recumbent after 8 to 10 h, convulsed, and died within 18 h of examination. She had been vaccinated against rabies in the spring. The clinician suspected viral encephalitis, probably EEE.

Fresh brain was submitted to the Animal Diseases Research Institute, Agriculture and AgriFood Canada, Nepean, Ontario, for rabies testing. Rabies viral antigen was not detected in the brain by the fluorescent antibody technique. Formalinized brain and fresh chilled brain were submitted to the Guelph Laboratory of the Veterinary Laboratory Services Branch, Ontario Ministry of Agriculture, Food and Rural Affairs, for routine histological examination and virus recovery.

Brain sections contained perivascular cuffs, mainly lymphocytes with a few neutrophils, around many vessels in white and gray matter of the cerebrum and cerebellum. A predominantly mononuclear infiltrate was present in the meninges. Blood vessels occasionally contained fibrin thrombi, and hemorrhage was common around vessels, especially in white matter. There was focal to locally extensive necrosis, predominantly of white matter, with lymphocytes, a few neutrophils, and cells with prominent karyorrhexis in necrotic foci in the cerebral cortex. A few axonal bodies were present in affected white matter.

Frozen brain was homogenized in a viral transport medium (Hanks' salts with 0.5% lactalbumin hydrolysate, 25 mM HEPES, penicillin, streptomycin, and nystatin at pH 7.2). Homogenates were clarified by centrifugation and the supernatant was filtered and inoculated onto partially confluent baby hamster kidney-21 (BHK-21), equine ovary, rabbit kidney-13, and African green monkey kidney cells. After adsorption, cell monolayers were rinsed and maintained in minimum essential medium with Earle's salts, supplemented with 2% fetal bovine serum. Monolayers were examined daily for cytopathic changes. Within 48 h of inoculation of the supernatant onto BHK-21 cell cultures, a cytopathic agent, suspected to be EEE virus, was recovered (designated isolate G92-03127). No viruses were recovered after 2 passages in the other cell lines.

Second passage virus was replicated in BHK-21 cells grown on glass coverslips, rinsed in phosphate-buffered saline (PBS) at pH 7.2, and fixed for 10 min in acetone on ice. Coverslips, with the cell monolayer up, were glued onto microscope slides, using Entellan (BDH, Toronto, Ontario), for fluorescent antibody staining. Acetone-fixed, control and infected, BHK-21 cells were forwarded to Health Canada, Laboratory Centre for Disease Control, Tunney's Pasture, Ottawa, Ontario, for definitive identification. Additional infected BHK-21 cells and supernatant were then prepared from the following virus strains: VEE; attenuated vaccine strain TC-83, derived from an epizootic virus isolated in Trinidad (9); WEE, strain 1406, from Manitoba, Canada; HJ, strain 72-666, from Connecticut, USA; and EEE, strain undesignated, from Quebec (7).

The acetone-fixed cells were tested against monoclonal antibodies 1B5C-3 and 1B1C-4 in fluorescent antibody assays. Monoclonal 1B5C-3 reacts only to North American variants, whereas 1B1C-4 reacts to both the North and South American variants of EEE virus (Karabatsos, personal communication). Cells on slides were incubated with the monoclonal antibodies, washed with phosphate-buffered saline (PBS), and incubated with fluorescein isothiocyanate-labelled goat antimouse IgG (Zymed, San Francisco, California, USA). The slides were then washed again, placed under mounting fluid (10% glycerol in PBS), and examined by ultraviolet microscopy. The G92-03127 isolate reacted with both (1B5C-3 and 1B1C-4) monoclonal antibodies, identifying it as a North American variant of EEE virus.

This identification was corraborated in a complement fixation (CF) test using hyperimmune mouse ascitic fluids produced to EEE, HJ, WEE, and VEE, and supernatants from isolates G92-03127, EEE, HJ, WEE, and VEE. This CF test was a modification of the microtiter method described by Sever (10) and used 2 units of antisheep hemolysin, 2 units of complement, 4 units of antigen, and a sheep red blood cell concentration of 0.4%. Using EEE hyperimmune ascitic fluid, isolate G92-03127 had an identical titer (1:128) to homologous EEE virus, with a reaction of <1:8 to HJ, WEE, and VEE monoclonal antibodies. Thus, G92-03127 was considered to be EEE virus.

Sera were subsequently collected from 9 in-contact horses and from 48 chickens, 30 ducks, and 3 geese housed in open-windowed facilities on the farm. Horse sera were tested for antibodies to EEE, WEE, VEE, and HJ viruses by a hemagglutination-inhibition (HI) test (11), modified to a microtiter technique (10). All sera were treated with acetone and absorbed with packed goose erythrocytes before HI testing. Four hemagglutinating units of each antigen were used. One horse had an HI titer of 1:320 to EEE virus, with no cross-reaction to the other alphavirus antigens. By CF test, this horse had a 1:32 titer to both the EEE virus from Quebec and isolate G92-03127. No antibodies (titer <1:8) were detected to WEE, VEE, or HJ viral antigens.

The avian sera were treated with acetone and protamine sulfate (Eli Lilly Canada Inc., Toronto, Ontario), absorbed with packed chicken erythrocytes (12) to remove hemagglutinin inhibitors, and tested by HI in microtiter plates for antibodies to EEE and WEE using 4 units of EEE and WEE antigens. Reactive sera were retested against the EEE, WEE, and HJ antigens. One duck serum was positive for HI antibody to WEE at 1:20 dilution and to HJ at 1:10 dilution; other birds were negative for HI antibodies to EEE and WEE.

This is the first confirmed case of EEE in a horse in Ontario since 1938 (6). The fever and characteristic clinical signs (1–3) suggested infection with EEE virus rather than rabies virus. The histopathological findings were consistent with a diagnosis of EEE (1). The isolation of EEE virus confirmed the diagnosis in the dead horse. The in-contact horse with antibody to EEE virus was apparently subclinically infected.

These 2 horses were pastured with access to a permanent hardwood swamp filled with water from summer rains. Horses pastured under such conditions are at greatest risk of EEE virus infection (3). Most cases in northern regions occur in rural areas during the mosquito season, between July and October (3). The farm birds that were housed a few hundred meters from the swamp, and could be regarded as sentinels, were HI antibody-negative to EEE virus, emphasizing the localized habitat of the vector.

The results of the avian serology are consistent with those of a recent EEE epizootic in Ohio (13) that occurred in a habitat similar to that of our case. In spite of the extent of the epizootic (19 equine cases), only 3 of 50 avian sera, representing 14 species, had antibodies to EEE virus; 4 other sera were marginally positive. The 2 horses on our farm may be sporadic infections, rather than the result of epizootic transmission. The single duck that was positive for antibodies to both WEE and HJ viruses suggests the presence of HJ virus activity, since there is no record of WEE virus in Ontario. Highlands J virus activity is commonly detected during EEE virus surveillance and disease studies (3). Clinical EEE did not occur on the same premises during the summer of 1993 or 1994.

This episode indicates the need for continued surveillance for EEE virus activity. This conclusion is supported by the recognition of 2 cases after 55 y without records of EEE disease; the activity of this virus in 3 states contiguous to Ontario; and the nature and similarity of the habitat to that of the Ohio outbreak.

Effective EEE virus vaccines are available to protect high-risk horses. Routine vaccination is not recommended in Canada because of the extremely low prevalence of disease. However, horses being transported to the eastern USA should be vaccinated before leaving Canada (1).

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